Diaryl Amidine Derivatives as Oncornaviral DNA Polymerase Inhibitors

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A total of 77 diaryl amidine derivatives were evaluated for their inhibitory effects on the DNA polymerase (reverse transcriptase) activity of Moloney murine leukemia virus. The amidino-substituted aryl moieties of the compounds consisted of phenyl, indole, indene, benzofuran, benzo[*b*]thiophene, or benzimidazole. Several of these compounds were found to inhibit oncornavirus-directed DNA synthesis with an ID_{50} of approximately 1 μg/mL. This ID_{50} was comparable to that of ethidium bromide, a compound which is considered to be a very effective inhibitor of oncornaviral DNA polymerases. Structure–function analysis revealed that the inhibitory activity of the diaryl amidine compounds depended on the planarity of the molecule, the presence of both amidino (or, preferably, imidazolino) groups, the absence of a central bridge or, if present, a short and rigid chain connecting the two aryl moieties, and, finally, the nature of these aryl residues (preferably benzofuran or indole). Hence, compounds 18 (4′,6-diimidazolino-2-phenylbenzo[*b*]thiophene) and 48 [5-amidino-2-(5-amidino-2-benzofuranyl)indole] featured among the most potent inhibitors of oncornavirus-directed DNA synthesis. For some representative diaryl amidines the kinetics of inhibition of viral DNA synthesis was investigated with $(A)_n$·oligo(dT) as template–primer and dTTP as substrate and assuming a quasi-steady-state reaction with a single substrate (dTTP), a single enzyme (reverse transcriptase), and a single product [$(A)_n$·$(dT)_n$]. Inhibition was not exactly competitive with respect to $(A)_n$·oligo(dT), although a purely competitive type of inhibition may have been expected if the compounds simply blocked the binding site(s) of the template–primer for the enzyme.

The discovery of reverse transcriptase (RNA-directed DNA polymerase) in oncogenic RNA viruses and various human tumor cells has prompted the search for substances which would be able to inhibit oncornaviral DNA synthesis, thereby preventing malignant transformation of the virus-infected cell.[1] As a consequence, a large number of reverse transcriptase inhibitors have been developed, which could, according to their mode of action,[2,3] be divided into the following classes: (i) enzyme-binding compounds, such as rifamycins, streptovaricins and pyran copolymer, which interact with the polymerase at a region other than the template-binding site; (ii), substrate analogues, such as 1-β-D-arabinofuranosylcytosine 5′-triphosphate (ara-CTP) and 5-mercapto-2′-deoxyuridine 5′-triphosphate; (iii) template analogues, such as poly(2′-azido-2′-deoxyadenylic acid), poly[2-(methylthio)adenylic acid],[4,5] and partially 5-thiolated poly(cytidylic acid), which compete with the normal RNA template for the template-binding site of the enzyme; (iv) polyanionic substances, such as suramin[6] and 5-tungsto-2-antimoniate,[7] which are not template analogues, yet inhibit the reverse transcriptase reaction in a manner that is competitive with the template–primer; (v) agents that chelate divalent cations, e.g., thiosemicarbazones and *o*-phenanthroline; and (vi) template-binding compounds, such as ethidium bromide, fagaronine, tilorone, anthracyclines (e.g., daunomycin), and distamycin A, which inhibit DNA polymerase activity through binding to specific base pairs, e.g., A-T, of the template–primer.

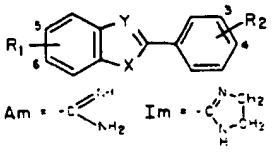
There are several inhibitors of DNA and RNA synthesis which are assumed to interact specifically with A-T base pairs. This class of compounds also includes 4′,6-diamidino-2-phenylindole (DAPI; 4), a diaryl amidine derivative that was originally synthesized by Dann et al.[8] and that has been shown to form fluorescent complexes with double-stranded DNA, viz., synthetic DNA duplexes containing A-T, A-U, and I-C base pairs.[9,10] DAPI strongly inhibits DNA and RNA polymerase activity but only with poly(dA-dT) as template; no inhibition is observed with poly(dG-dC) as template.[11,12] DAPI and some of its congeners (6, 7, and 15) have also been reported to inhibit the endogenous RNA-directed DNA polymerase associated with Friend murine leukemia virus, although the extent of inhibition obtained was quite modest.[3] We have now evaluated DAPI and 76 other diaryl amidine derivatives for their inhibitory effects on oncornaviral DNA polymerase activity, using Moloney murine leukemia virus as source of the enzyme. These studies were aimed at (i) delineating the structural features that govern the antioncornaviral DNA polymerase activity of diaryl amidine derivatives and (ii) at gaining further insight in their mechanism of action at the molecular level. Analysis of the structure–function relationship will obviously orient our efforts toward the synthesis of new and more potent inhibitors of oncornaviral DNA polymerase.

It should be pointed out that the DNA synthesis measured in our assay conditions may have been the result of two successive DNA polymerase reactions: an RNA-directed DNA polymerase (reverse transcriptase) reaction leading to the synthesis of single-stranded complementary DNA (ss cDNA) and a DNA-directed DNA polymerase reaction that would convert the ss cDNA into double-stranded (ds) cDNA. Where DNA synthesis was measured

(1) Chandra, P.; Ebener, U.; Steel, L. K.; Laube, H.; Götz, A. "Advances in Cancer Research": Gallo, R. C., Ed.; CRC Press: Cleveland, Ohio, 1977; pp 167-187.
(2) Smith, R. G.; Gallo, R. C. *Life Sci.* 1974, *15*, 1711.
(3) Chandra, P.; Steel, L. K.; Ebener, U.; Woltersdorf, M.; Laube, H.; Kornhuber, B.; Mildner, B.; Götz, A. *Pharmacol. Ther., Part A*, 1977, *1*, 231.
(4) De Clercq, E. "Antiviral Mechanisms in the Control of Neoplasia"; Chandra, P., Ed.; Plenum Press: New York, 1979; pp 539–551.
(5) De Clercq, E.; Fukui, T.; Kakiuchi, N.; Ikehara, M.; Hattori, M.; Pfleiderer, W. *Cancer Lett.* 1979, *7*, 27.
(6) De Clercq, E. *Cancer Lett.* 1979, *8*, 9.
(7) Chermann, J.-C.; Sinoussi, F. C.; Jasmin, C. *Biochem. Biophys. Res. Commun.* 1975, *65*, 1229.
(8) Dann, O.; Bergen, G.; Demant, E.; Volz, G. *Justus Liebigs Ann. Chem.* 1971, *749*, 68.
(9) Kapuscinski, J.; Szer, W. *Nucleic Acids Res.* 1979, *6*, 3519.
(10) Kapuscinski, J.; Yanagi, K. *Nucleic Acids Res.* 1979, *6*, 3535.
(11) Chandra, P.; Mildner, B.; Dann, O.; Metz, A. *Mol. Cell. Biochem.* 1977, *18*, 81.
(12) Mildner, B.; Metz, A.; Chandra, P. *Cancer Lett.* 1978, *4*, 89.

0022-2623/80/1823-0787$01.00/0

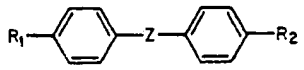
Table I. Class A Compounds



no.	X	Y	R_1	R_2	oncornaviral DNA polymerase ID_{50},[c] μg/mL	ref for synth
			I. indoles			
1	NH	CH	Am[a] (5)	Am (3)	4	13
2	NH	CH	Am (5)	Am (4)	8	13
3	NH	CH	Am (6)	Am (3)	8	13
4[d]	NH	CH	Am (6)	Am (4)	5	13
5	NH	CNH_2	Am (6)	Am (4)	12	18
6	NH	CH	Im[b] (6)	Im (4)	6	19
			II. benzofurans			
7	O	CH	Am (5)	Am (4)	12	13
8	O	CH	Im (5)	Im (4)	4.5	20
9	O	CH	Am (5)	NH_2 (4)	270	14
10	O	CH	$NHC(=NH)NH_2$ (5)	$NHC(=NH)NH_2$ (4)	8	21
11	O	CH	$N=CHN(CH_3)_2$ (5)	$N=CHN(CH_3)_2$ (4)	40	21
			III. benzo[*b*]thiophenes			
12	S	CH	Am (5)	Am (3)	6	15
13	S	CH	Am (5)	Am (4)	5	15
14	S	CH	Am (6)	Am (3)	8	15
15	S	CH	Am (6)	Am (4)	4	15
16	S	CNH_2	Am (5)	Am (4)	8	19
17	S	CH	Im (5)	Im (4)	2.8	14
18	S	CH	Im (6)	Im (4)	1.2	20
19	S	CH	$NHC(=NH)NH_2$ (5)	$NHC(=NH)NH_2$ (4)	11	14
20	S	CH	$NHC(=NH)NH_2$ (6)	$NHC(=NH)NH_2$ (4)	6	14
21	S	CH	$CH=NNHC(=NH)NH_2$ (6)	$CH=NNHC(=NH)NH_2$ (4)	2	14
22	SO_2	CH	Am (5)	Am (4)	11	15
23	SO_2	CH_2	Am (5)	Am (4)	90	15
			IV. indene			
24	CH_2	CH	Am (6)	Am (4)	8	13

[a] For structure of amidino (Am), see structure over column heads. [b] For structure of imidazolino (Im), see structure over column heads. [c] ID_{50} values were calculated after a 120-min incubation of the reaction mixtures (see Experimental Section). All experiments were done in duplicate or triplicate. [d] DAPI.

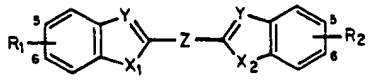
Table II. Class B Compounds



no.	Z	R_1	R_2	oncornaviral DNA polymerase ID_{50},[c] μg/mL	ref for synth
25[d]	CH=CH	Am[a]	Am	12	
26[e]	NHN=N	Am	Am	11	
27[f]	$O(CH_2)_5O$	Am	Am	100	
28		Am	Am	6.5	16
29		Am	Am	10	16
30		Am	Am	6	16
31		Am	Am	28	16
32		Am	Am	1.2	16
33	*p*-NHCO-C_6H_4-CONH	Am	Am	>400	22
34	*p*-NHCO-C_6H_4-CONH	Im[b]	Im	15–200	22

[a-c] See corresponding footnotes to Table I. [d] 25 = stilbamidine isethionate. [e] 26 = berenil. [f] 27 = pentamidine isethionate.

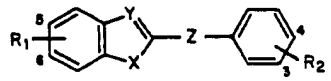
Table III. Class C Compounds



no.	X_1	Y	X_2	Z	R_1	R_2	oncornaviral DNA polymerase ID_{50},[c] μg/mL	ref for synth
				I. benzofuran/benzofuran				
35	O	CH	O	CH_2	Am[a] (5)	Am (5)	14	17
36	O	CH	O	$(CH_2)_2$	Am (5)	Am (5)	12	17
37	O	CH	O	CH=CH	Am (5)	Am (5)	1.3	17
38	O	CH	O	CH=CH	Am (5)	Am (6)	1.2	23
39	O	CH	O	CH=CH	Am (6)	Am (6)	1.6	23
40	O	CCH_3	O	CH=CH	Am (5)	Am (5)	8	19
41	O	CH	O	$CH=CCH_3$	Am (5)	Am (5)	14	17
42	O	CH	O	CH_2CHCH_3	Am (5)	Am (5)	15	17
43	O	CH	O	$(CH=CH)_2$	Am (5)	Am (5)	3	21
44	O	CH	O	$(CH=CH)_2$	Am (5)	Am (5)	1.6	21
45	O	CH	O		Am (5)	Am (5)	2	16
46	O	CH	O	CH=CH	Im[b] (5)	Im (5)	1.1	20
47	O	CH	O	$(CH=CH)_2$	Im (5)	Im (5)	1	21
				II. benzofuran/indole				
48	O	CH	NH		Am (5)	Am (5)	0.9	17
49	O	CH	NH	CH=CH	Am (5)	Am (5)	1.4	23
50	O	CH	NH	CH=CH	Am (5)	Am (6)	1.1	23
				III. benzofuran/benzo[*b*]thiophene				
51	O	CH	S	CH=CH	Am (5)	Am (5)	4	17
				IV. benzo[*b*]thiophene/benzo[*b*]thiophene				
52	S	CH	S	CH=CH	Am (5)	Am (5)	11	17
				V. benzimidazole/benzimidazole				
53	NH	N	NH	CH=CH	Am (5)	Am (5)	100	17

[a-c] See corresponding footnotes to Table I.

Table IV. Class D Compounds



no.	X	Y	Z	R_1	R_2	oncornaviral DNA polymerase ID_{50},[c] μg/mL	ref for synth
			I. indoles				
54	NH	CH	CH=CH	Am[a] (6)	Am (4)	6	24
55	NH	CH	C_6H_4-O	Am (6)	Am (4)	3-250	16
56	NH	CH	C_6H_4-O	Am (6)	Am (3)	6	20
57	NH	CH	CH=CH-C_6H_4-O	Am (6)	Am (4)	1.4	20
58	NH	CH	C_6H_4-O	Im[b] (6)	Im (3)	3	20
59	NH	CH	C_6H_4-O	Im (6)	Im (4)	1.4	20
60	NH	CH	CH=CH-C_6H_4-O	Im (6)	Im (4)	1.6	20
			II. benzofurans				
61	O	CH	CH=CH	Am (5)	Am (4)	8	14
62	O	CH	$(CH_2)_2$	Am (5)	Am (4)	120	14
63	O	CH	$CH=CCH_3$	Am (5)	Am (4)	4	14
64	O	CH	CONH	Am (5)	Am (4)	7.5	23
65	O	CH	CH=NNH	Am (5)	Am (4)	7.5	21
66	O	CH	$(CH=CH)_2$	Am (5)	Am (4)	0.9	21
67	O	CH	$(CH=CH)_2$	Am (5)	Am (4)	1.6	21
68	O	CH	CH=CH-C_6H_4-O	Am (5)	Am (4)	14	21
69	O	CH	C_6H_4-N=NNH	Am (5)	Am (4)	1.3	14
70	O	CH	CH=CH	Im (5)	Im (4)	6	21
71	O	CH	$(CH=CH)_2$	Im (5)	Im (4)	4	21
72	O	CH	CONH	Am (5)	Im (4)	10	23
73	O	CH	CH=CH-C_6H_4-O	Im (5)	Im (4)	9	21
			III. benzo[*b*]thiophene				
74	S	CH	CONH	Am (5)	Am (4)	1.5	14

[a-c] See corresponding footnotes to Table I.

in the absence of an exogenous template–primer after a 120-min incubation (Tables I–V; Figure 1a–d), as was regularly done in our structure–function relationship studies, both reactions may have occurred, and the ID_{50}

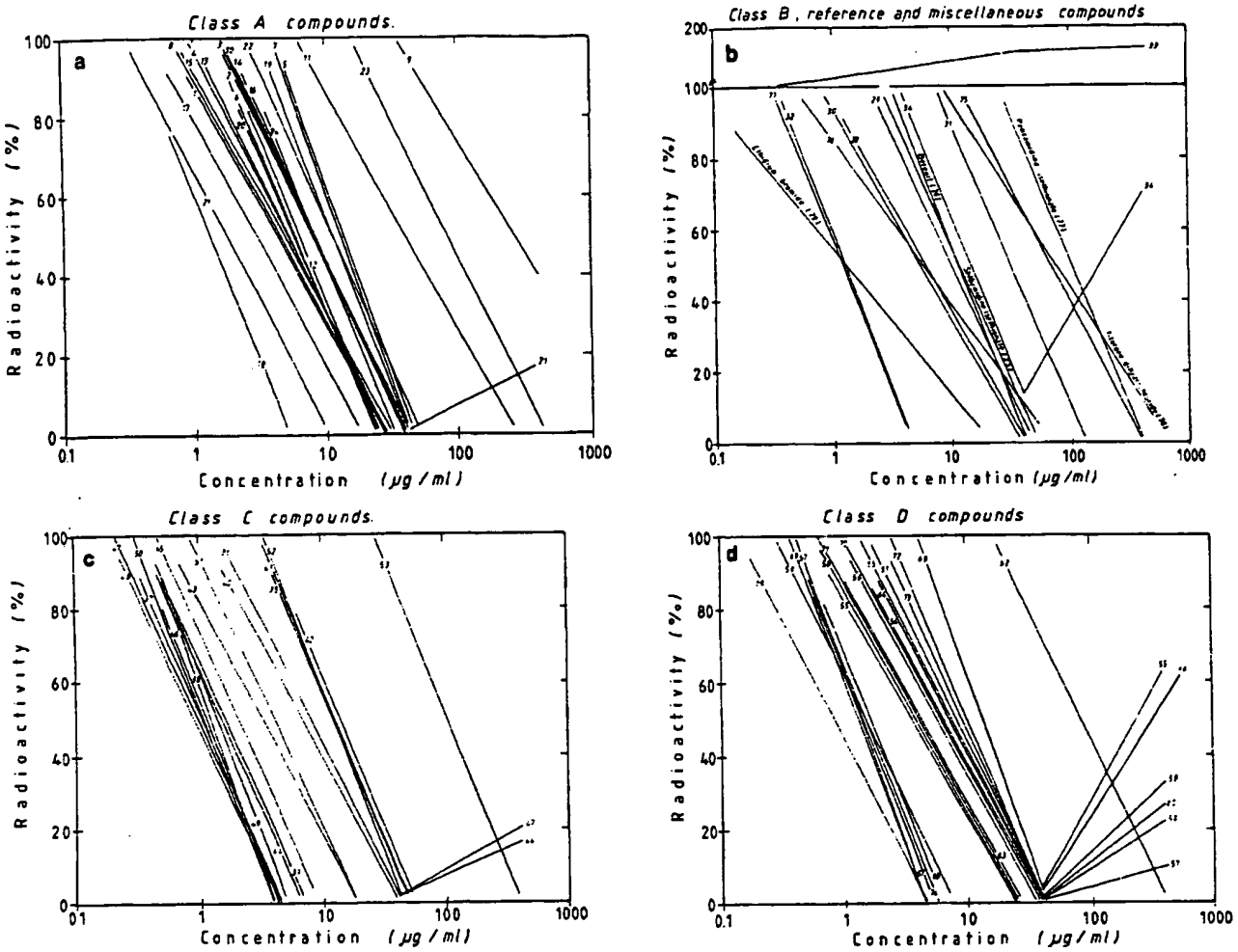


Figure 1. Dose–response relationship of inhibition of oncornaviral DNA polymerase diaryl amidine derivatives. The endogenous RNA (of Moloney murine leukemia virus) served as the template of the DNA polymerase reaction, and DNA synthesis was monitored by [*methyl*-^{3}H]dTMP incorporation (see Experimental Section). The reaction mixtures were incubated for 120 min: (a) class A compounds; (b) class B, reference and miscellaneous compounds; (c) class C compounds; (d) class D compounds.

(50% inhibitory dose) values obtained in these conditions may reflect the ID_{50} for both DNA polymerase reactions combined. Where DNA synthesis was measured in the presence of $(A)_n$-oligo(dT) as template–primer after a 15-min incubation (Figure 2a–f), as was regularly done in our enzyme kinetics studies, only the reverse transcriptase reaction may have occurred, and the inhibitory activity noted in these conditions may be interpreted as true anti-reverse-transcriptase activity.

Chemistry. The amidino and imidazolino compounds were prepared by the well-known Pinner reaction from the corresponding nitriles. These nitriles were obtained by a cyanido exchange with copper(I) cyanide from the corresponding bromo compounds having the completed ring structures. The different classes of ring structures were synthesized as described previously.[13-24] References to the synthesis of the individual compounds are provided in Tables I–V.

Results and Discussion

Structure–Function Relationship. Depending on the nature of the amidino-substituted ring structures, the compounds were divided into five classes (Tables I–V). Class D (Table IV) can actually be regarded as an extension of class A (Table I) compounds in which the amidino-substituted rings are linked by a carbon (or carbon–nitrogen) chain of variable length. Classes A, C, and D were further subdivided according to the type of the heterocyclic ring (indole, benzofuran, benzo[*b*]thiophene, indene, or benzimidazole) (Tables I, III, and IV). Class

(13) Dann, O.; Bergen, G.; Demant, E.; Volz, G. *Justus Liebigs Ann. Chem.* 1971, *749*, 68.
(14) Dann, O.; Fernbach, R.; Pfeifer, W.; Demant, E.; Bergen, G.; Lang, S.; Lürding, G. *Justus Liebigs Ann. Chem.* 1972, *760*, 37.
(15) Dann, O.; Hieke, E.; Hahn, H.; Miserre, H. H.; Lürding, G.; Rössler, R. *Justus Liebigs Ann. Chem.* 1970, *734*, 23.
(16) Dann, O.; Fick, H.; Pietzner, B.; Walkenhorst, E.; Fernbach, R.; Zeh, D. *Justus Liebigs Ann. Chem.* 1975, *1975*, 160.
(17) Dann, O.; Volz, G.; Demant, E.; Pfeifer, W.; Bergen, G.; Fick, H.; Walkenhorst, E. *Justus Liebigs Ann. Chem.* 1973, *1973*, 1112.
(18) Tidwell, R. R.; Geratz, J. D.; Dann, O.; Volz, G.; Zeh, D.; Loewe, H. *J. Med. Chem.* 1978, *21*, 613.
(19) Dann, O.; Volz, G. *Justus Liebigs Ann. Chem.* 1980, in press.
(20) Dann, O.; Ruff, J. *Justus Liebigs Ann. Chem.* 1980, in press.
(21) Dann, O.; Griessmeier, H. *Justus Liebigs Ann. Chem.* 1980, in press.
(22) Wander, A.; British Patent 1007-334, 1965; *Chem. Abstr.* 1966, *64*, 5102 [compound 33 was prepared anew by J. Ruff, whereas compound 34 was a gift from Dr. R. Fischer (Wander A.G., Bern)].
(23) Dann, O.; Char, H. *Justus Liebigs Ann. Chem.* 1980, in press.
(24) Dann, O.; Wolff, H. P. *Justus Liebigs Ann. Chem.* 1980, in press.

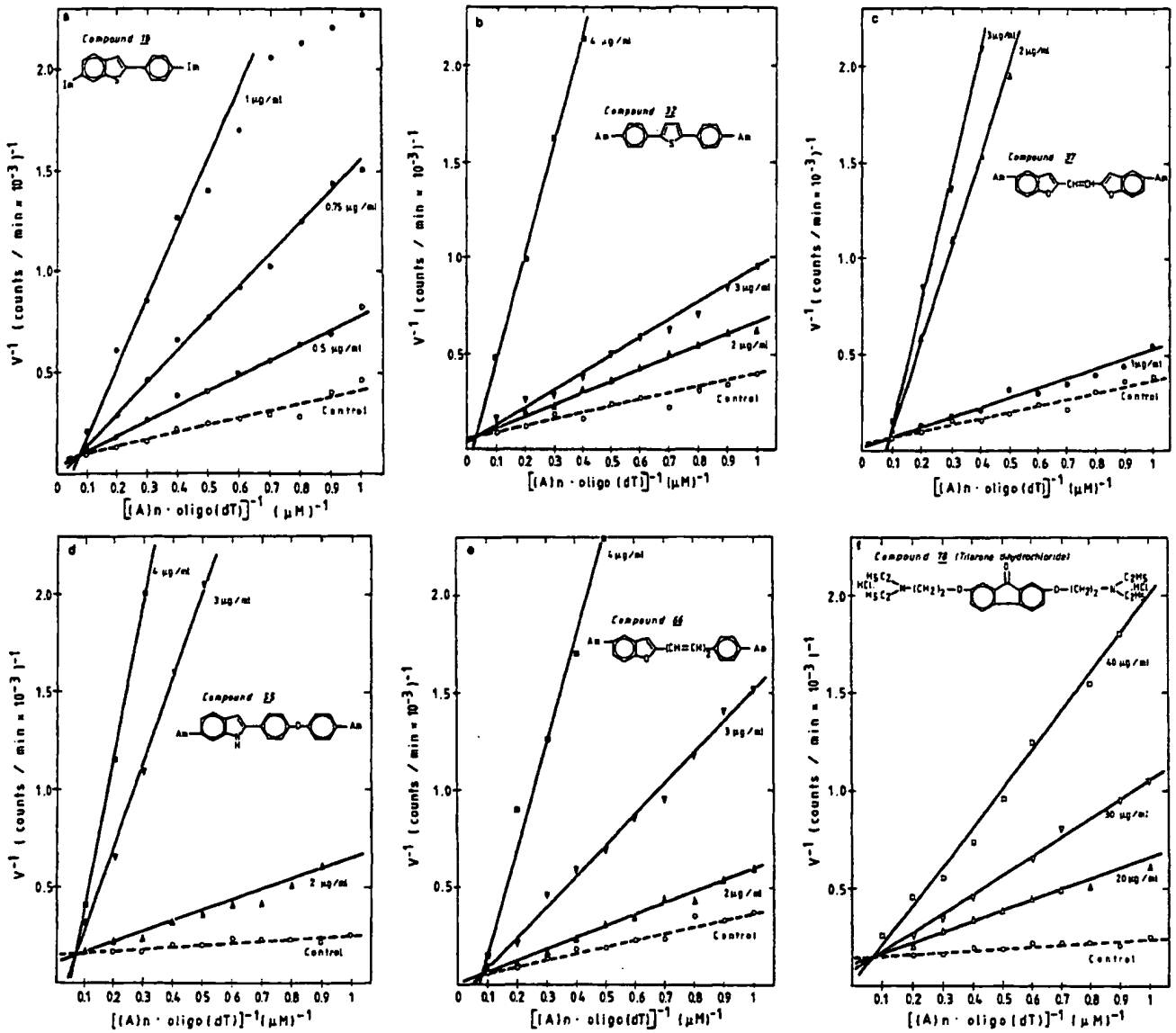


Figure 2. Lineweaver-Burk plots for the kinetics of inhibition of oncornaviral DNA polymerase by diaryl amidine derivatives. $(A)_n \cdot (dT)_{12-18}$ served as the template of the DNA polymerase reaction, and DNA synthesis was monitored by [*methyl*-^{3}H]dTMP incorporation (see Experimental Section). The reaction mixtures were incubated for 15 min: (a) compound 18; (b) compound 32; (c) compound 37; (d) compound 55; (e) compound 66; (f) compound 78 (tilorone dihydrochloride).

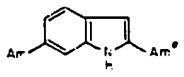
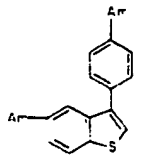
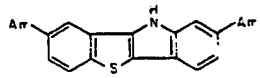
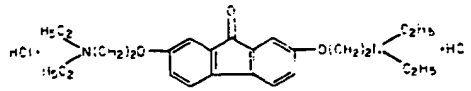
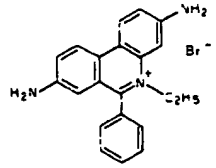
B (Table II) contained those compounds where both of the amidino-substituted rings was a benzene [e.g., stilbamidine (25), berenil (26), and pentamidine (27)]. Some miscellaneous compounds, including the reference materials tilorone [2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one, 78] and ethidium bromide (79), were brought together in Table V.

All compounds tested are listed in Tables I–V with their respective ID_{50} values for oncornaviral DNA polymerase activity and the references for their syntheses. The ID_{50} (50% inhibitory dose) values were calculated for the DNA synthesis measured after 120-min incubation of the reaction mixtures (see Experimental Section). In these assays the endogenous viral RNA served as template for the DNA polymerase reaction and, as mentioned above, it is likely that the DNA synthesis measured at 120 min was the result of two successive DNA polymerization cycles (RNA directed and DNA directed).

The dose–response curves obtained for the different diaryl amidine derivatives when incubated with the DNA polymerase assay mixture for 120 min at 37 °C are presented in Figures 1a (class A compounds), 1b (class B, reference and miscellaneous compounds), 1c (class C compounds), and 1d (class D compounds).

Within **class A compounds** (Table I; Figure 1a) the highest antipolymerase activity was noted for compound 18, which was about four times more active than the standard diaryl amidine DAPI (4). This increase in activity was probably due to substitution of amidino by imidazolino groups and not to substitution of the ring nitrogen by sulfur. Indeed, the benzo[*b*]thiophene analogue of DAPI (compound 15) did not markedly differ in activity from DAPI itself. Neither did substitution of the ring nitrogen by carbon (compound 24) result in a substantial change of activity. Similarly, little, if any, differences were observed in DNA polymerase inhibiting potency between the indole, benzofuran, and benzo[*b*]thiophene derivatives 2, 7, and 13 and between the indole and benzo[*b*]thiophene derivatives 3 and 14. Thus, isosteric modifications did not have a significant influence on the capacity of class A compounds to act as oncornaviral DNA polymerase inhibitors.

Table V. Miscellaneous Compounds

no.	structure	oncornaviral DNA polymerase ID_{50},[c] μg/mL	ref for synth
75		70	20
76		6	14
77		1.2	17
78[d]		75	
79[e]		1.2	

[a,c] See corresponding footnotes to Table I. [d] 78 = tilorone dihydrochloride. [e] 79 = ethidium bromide.

On the other hand, substitution of imidazolino for amidino generally led to an appreciable increase in antipolymerase activity (compare compounds 7 with 8, 13 with 17, and 15 with 18). Whereas substitution of the amidino groups by guanidino groups (compounds 10 and 7) slightly increased the inhibitory activity, a three- to fourfold decrease in activity was noted upon introduction of N=CH-$N(CH_3)_2$ groups (compound 11). If one of the amidino groups was replaced by amino (compounds 9 and 7), there was almost a complete loss of inhibition of DNA polymerase activity. Thus, both amidino (imidazolino or guanidino) groups appear to be necessary for optimal inhibitory activity.

Molecular planarity seems to be equally important for inhibitory activity. If the planar state of compound 13 was reduced by a 1,1-dioxy substitution (compounds 22 and 23) and hydrogenation of carbons 2 and 3 (compound 23), there was a concomitant reduction in inhibitory activity.

Within **class B and the miscellaneous compounds** (Tables II and V; Figure 1b), compounds 32 and 77 displayed the greatest inhibitory effects on oncornaviral DNA polymerase activity. The ID_{50} values recorded for compounds 32 and 77 were identical with that of ethidium bromide (79), although the slopes of the dose–response curves of compounds 32 and 77 markedly differed from the slope of the ethidium's dose–response curve. Two other trypanocidal drugs, stilbamidine (25) and berenil (26), were approximately 10 times less effective as oncornaviral DNA polymerase inhibitors than ethidium, whereas a fourth established trypanocide, pentamidine (27), was even 100 times less active. Apparently, the connecting chain of compound 27 is too flexible to sustain optimal activity. The central chain of compound 33 proved totally incompatible with anti-DNA polymerase activity, although the deleterious effect of this particular chain could be partially overcome by substituting imidino for amidino in the phenyl side rings (compound 34). Characteristically, compound 34 exhibited its maximum inhibitory activity at a concentration of about 40 μg/mL; at higher concentrations it lost its inhibitory activity.

Although tilorone (78) has been widely publicized as a potent inhibitor of oncornaviral DNA polymerases,[25-28] it did not inhibit DNA polymerase activity in our assay system unless fairly high concentrations (40–400 μg/mL) were employed. With an ID_{50} of 75 μg/mL, tilorone ranked among the least active compounds tested. It should be pointed out, however, that the ID_{50} values presented in Tables I–V were obtained from DNA polymerase assays where the endogenous viral RNA served as template for the enzyme, whereas in previous assays, where tilorone caused a relatively more potent inhibition of DNA polymerase activity, exogenous templates, such as poly(dA-dT), were added to the reaction mixtures.[25,27,28] The inhibitory effects noted with tilorone under these conditions may be attributed to a direct interaction of the drug with the synthetic template, since tilorone specifically binds to dA-dT regions.[29]

Within **class C** (Table III; Figure 1c), various compounds (37–39, 44, 46–50) excelled in anti-DNA polymerase activity so that, as a whole, this class of compounds or at least the bis(amidinobenzofuran) and amidino(amidinobenzofuranyl)indole derivatives could be considered as the diamidine compounds with the greatest anti-DNA polymerase potentials.

The positioning of the amidino groups did not markedly influence the inhibitory effects on oncornaviral DNA polymerase activity: for instance, there was little, if any, change in inhibitory activity if the amidino group of one or both benzofuran rings was moved from position 5 (compound 37) to position 6 (compounds 38 and 39). Neither was there an appreciable difference in activity

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between the positional isomers of indole (compounds 49 and 50). As noted above for class A and class B compounds, substitution of imidazolino for amidino brought about an increase in inhibitory activity (as demonstrated by compounds 46 and 47 relative to compounds 37 and 43, respectively).

The inhibitory activity of the bis(amidinobenzofuran) derivatives was critically influenced by the nature of the bridge connecting the two amidino-substituted rings: most effective were the compounds with an ethene bridge (e.g., 37–40, 46, and 49–51). A substantial decrease in activity was observed in advancing from the ethene derivative (37) to the methane (35), ethane (36), isopropene (41), or isopropane (42) derivative. Lengthening of the connecting ethenyl chain by one or two ethenyl units did not cause drastic changes in activity (compare compound 37 with compounds 43 and 44). The slightly increased activity of compound 48 as compared to that of 49 suggests that a connecting chain is even not necessary for optimal inhibitory activity.

Whereas isosteric replacements did not cause major changes in activity within class A compounds, such substitutions had more dramatic consequences for the class C compounds. For example, substitution of sulfur for oxygen in one or both benzofuran rings resulted in a three- to eightfold decrease of anti-DNA polymerase activity (compounds 37, 51, and 52). Modification of the benzofuran rings to benzimidazole rings led to an 80-fold decrease of activity (compounds 37 and 53).

Within **class D compounds** (Table IV; Figure 1d), maximal inhibitory activity was observed for compound 66, whereas, based on previous considerations (see discussion of class C compounds), either compound 61, 70, or 71 may have been expected to be a more potent oncornaviral DNA polymerase inhibitor than 66. Compound 71 was about four times less active than compound 66: this is a rather unique example of reduction in activity upon substitution of imidazolino for amidino. In most other cases, the imidazolino derivatives proved more effective than the corresponding amidino derivatives (compare compounds 55 with 59, 56 with 58, 61 with 70, and 68 with 73).

As noted above for the class C compounds, there was a substantial decrease in anti-DNA polymerase activity if the ethene bridge was replaced by an ethane (compounds 61 and 62), but, if the connecting chain was extended by one or two ethenyl units, there was an increased inhibitory activity (compounds 61, 66, and 67).

In some instances, isosteric replacements effected rather significant changes in activity: for instance, a 10-fold decrease in anti-DNA polymerase activity was witnessed in going from the indole to the benzofuran series (compounds 57 and 68); this effect was probably due to substitution of oxygen for nitrogen in the indole ring, since the concomitant shift of the amidino group from position 6 to 5 is not expected to impose such a change in activity (see discussion of class C compounds). Another example of isosteric replacement is the substitution of sulfur for oxygen in compound 64. The resulting benzo[*b*]thiophene derivative 74 gained a fivefold increase in activity. This contrasts with our findings for the class C compounds where the benzo[*b*]thiophene and mixed benzofuran–benzo[*b*]thiophene derivatives were less active than the benzofuran derivative (compounds 37, 51, and 52).

As occasionally observed for some class A, B, and C compounds (21, 34, 44, and 47), several class D compounds (55, 57–59, 60, and 68) partially lost their inhibitory effects on oncornaviral DNA polymerase activity when assayed at supraoptimal concentrations (400 μg/mL) (Figure 1a–d). Although the exact mechanism of this curious phenomenon remains to be elucidated, it points to a dual effect in the mode of action of the diaryl amidine derivatives, a first one that leads to an inhibition of DNA polymerase activity and a second one that actually stimulates DNA polymerase activity. Both effects may be based on the specific manner(s) by which the diaryl amidine derivatives interact with the RNA (and DNA) template(s) of the DNA polymerases.

Kinetics of Enzyme Inhibition. The kinetics of inhibition of the oncornaviral DNA polymerase reaction by diaryl amidine derivatives were studied in the presence of the synthetic homopolymer–oligomer $(A)_n$·$(dT)_{12-18}$ as template–primer.[30-33] $(A)_n$·oligo(dT) is a more efficient, although less specific, template–primer for RNA tumor virus RNA-directed DNA polymerases than $(C)_n$·oligo(dG) or $(Cm)_n$·oligo(dG) [$(Cm)_n$ being poly(2′-*O*-methylcytidylic acid)].[34-38] Enzyme kinetics were determined, assuming a quasi-steady-state for the enzyme intermediates, and the reaction conditions [incubation time, $(A)_n$·oligo(dT) concentrations] were chosen so as to keep the reaction first order with respect to the template–primer. It was also assumed that under our test conditions a single reaction occurred with dTTP as substrate, reverse transcriptase as enzyme, and $(dT)_n$ as product.

From each class of diaryl amidine derivatives, one or two representative compounds were chosen to establish their kinetics of inhibition of the $(A)_n$·oligo(dT)-directed DNA polymerase reaction. As evidenced by Lineweaver–Burk plots (Figure 2a–f), the inhibition of DNA polymerase by compounds 18 (Figure 2a), 32 (Figure 2b), 37 (Figure 2c), 55 (Figure 2d), and 66 (Figure 2e) was not exactly competitive with respect to $(A)_n$·oligo(dT). A similar, *quasi-competitive* type of inhibition was also obtained for tilorone (78) (Figure 2f), which suggests that tilorone binds to $(A)_n$·oligo(dT) in the same manner as the diaryl amidine derivatives. A purely competitive type of inhibition could be anticipated if the compounds simply inactivated the template–primer regions that normally act as binding site(s) for the DNA polymerase. Such pure competitive inhibition has been previously reported for the inhibitory effect of DAPI (4) on the DNA polymerase and RNA polymerase of bacterial origin, both evaluated with poly(dA-dT) as template.[11,12]

In line of a competitive inhibition type, inhibition of the oncornaviral DNA polymerase by the diaryl amidines 18, 32, 37, 55, and 66 and by tilorone (78) could be overcome by progressively increasing the $(A)_n$·oligo(dT) concentration (Figure 2a–f), but, whereas a competitive inhibitor does not normally alter the maximal velocity of an enzymatic reaction, in our assays V_{max} tended to increase with increasing concentrations of the diaryl amidines (and tilorone). In this sense, the kinetics of inhibition by tilorone and the diaryl amidine compounds differed from that of ethidium bromide (79), where the inhibition was intermediary between competitive and noncompetitive and

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where the V_{max} gradually decreased with increasing concentrations of the inhibitor.

The aberrant behavior of the diaryl amidines (and tilorone), as reflected by their enhancing effect on V_{max} (Figure 2a–f), may seem related to the biscationic nature of the compounds, which at a given polynucleotide–drug ratio may actually stimulate the template efficiency of both natural (viral) and synthetic RNAs [$(A)_n$]. This "secondary" effect may also contribute to a loss of inhibitory potency at higher concentrations, as has been witnessed for several diaryl amidine derivatives (Figure 1a–d). It would appear therefore as though diaryl amidines could interact with the RNA-directed (reverse) transcriptase template in a bimodal fashion, resulting in either a switch on or switch off of the template function. In how far these interactions involve intercalation between two successive base pairs and/or sidelong binding to the phosphate residues remains obviously a matter of further study.

In Vivo Tests. Preliminary findings indicate that diaryl amidines may also be effective in inhibiting tumor development, and mortality associated therewith, in mice inoculated intramuscularly with Moloney murine sarcoma virus. In a single experiment, 10 out of 10 NMRI mice died within 2 months after infection with the oncornavirus; however, if they had been treated with diaryl amidine **37** (250 μg/50 μL injected intraperitoneally at 4 h before and 1, 2, 3, and 4 days after virus inoculation), 5 out of 10 mice were still alive 2 months after virus inoculation (J. Balzarini, E. De Clercq, and O. Dann, unpublished data, 1979). While these findings suggest that diaryl amidine derivatives should be further explored for their antitumor potentials, it remains to be established whether their inhibitory effects on Moloney sarcoma virus-induced tumor growth are mediated by an inhibition of oncornaviral DNA synthesis.

Conclusions

Diaryl amidines, viz., DAPI (**4**), are assumed to interact specifically with A-T regions of DNA or DNA·RNA hybrids.[11,12] There are some other amidine derivatives, e.g., distamycin A, which are also known to bind preferentially to A-T rich regions of DNA and which, akin to diaryl amidines, inhibit DNA polymerase activity associated with RNA tumor viruses.[37–39] However, distamycins do so at concentrations (100 μg/mL)[38,39] which are about 100-fold higher than those at which the most active diaryl amidines inhibit DNA polymerase activity. These diaryl amidines also surpass various other oncornaviral DNA polymerase inhibitors, including tilorone (**78**), in inhibitory potency. The extent of their activity is comparable to that of ethidium bromide (**79**), one of the most effective inhibitors of oncornaviral DNA polymerase reported to date.[6]

From a general structure–function standpoint one may infer that inhibitory activity of the diaryl amidine derivatives depended on the planar state of the molecule, the presence of both amidino (or, preferably, imidazolino) groups, and the presence of a short and rigid chain connecting the two aryl moieties. The nature of these aryl residues seemed less critical, although the most active molecules were found among those diaryl derivatives that contained one or (preferably) two benzofuran or indole moieties (e.g., compounds **46–48** and **50**).

Previous studies[18] have indicated that the antiproteolytic activity of diaryl amidine derivatives does not correlate with their anticoagulant activity and that even within the antiprotease activity marked differences occur, depending on the protease tested (trypsin, trombin, or kallikrein). A comparison of our structure–function data with those previously reported by Tidwell et al.[18] reveals that there is no direct correlation between anti-protease activity and anti-DNA polymerase activity. For example, compound **8** was inactive as a trypsin inhibitor, yet fairly active as a oncornaviral DNA polymerase inhibitor. Whereas substitution of imidazolino for amidino generally increased the anti-DNA polymerase potency (e.g., compare compound **7** with **8**, **13** with **17**, etc.), it almost annihilated the antiprotease activity (compare compound **14** with **17** and compound **24** with **32** in ref 18). Some very effective trypsin inhibitors (compound **29** in ref 18) were barely active as a DNA polymerase inhibitor (compound **23** in Table I). Another effective protease inhibitor (compound **21** in ref 18) was by far the most potent anticoagulant agent, yet is proved to be only average in its ability to inhibit DNA polymerase activity (compound **36** in Table III). These differences obviously point to be specificities of the compounds concerned, be it as protease inhibitor or DNA polymerase inhibitor. They also justify further explorative studies with those compounds that demonstrate the greatest specificity and potency as oncornaviral DNA polymerase inhibitors.

Experimental Section

DNA Polymerase Assays. For those assays where the endogenous viral RNA served as template (see section "Structure–Function Relationship"), the reaction mixtures (250 μL) contained 40 mM Tris-HCl (pH 7.8), 50 mM NaCl, 4 mM $MnCl_2$, 1.6 mM dithiothreitol, 0.0125% (v/v) Triton X-100, 0.64 mM each of dATP, dCTP, and dGTP, 10 μCi (0.33 mmol) of [*methyl*-^{3}H]dTTP, 20 μL of virus stock suspension, and varying concentrations of the compounds (0.4, 4, 40, and 400 μg/mL). Where the stock solutions of the compounds had been prepared in Me_2SO, control assays were run containing the corresponding amounts of Me_2SO. The reaction mixtures were incubated at 37 °C for 0, 30, 60 or 120 min, at which time 50-μL aliquots were withdrawn and tested for acid-insoluble radioactivity.[40]

For those assays where $(A)_n$·oligo(dT) served as template–primer (see section "Mechanism of Action"), the reaction mixtures (100 μL) contained 25 mM Tris-HCl (pH 8.3), 100 mM NaCl, 0.5 mM $Mn(OOCCH_3)_2$, 5 mM dithiothreitol, 0.04% (v/v) Triton X-100, 5.0 μCi (0.16 nmol) of [*methyl*-^{3}H]dTTP, 5 μL of virus stock suspension, varying concentrations of the template–primer [$(A)_n$ at 1.0, 1.11, 1.25, 1.43, 1.66, 2.0, 2.5, 3.3, 5.0, or 10 μM; $(dT)_{12-18}$ at one-fourth the concentration of $(A)_n$], and varying concentrations of the compounds (as indicated in the figures). The reaction mixtures were incubated at 37 °C for 15 min, at which time acid-insoluble radioactivity was measured.[40]

Stock solutions of the compounds were prepared at 10 mg/mL in Me_2SO or at 10, 1, 0.1 or 0.01 mg/mL in twice distilled water. All stock solutions were stored at 4 °C until used.

[*methyl*-^{3}H]dTTP was purchased from the Radiochemical Centre, Amersham England, through IRE (Institut des Radio-Eléments), Fleurus, Belgium. The specific radioactivity of the radiochemical was 30 Ci/mmol.

Moloney murine leukemia virus was obtained from Electro-Nucleonics Laboratories, Bethesda, Md. (catalog no. 1024; lot no. 719-24-9, 810-71-8, 5004-26-56, 5014-40-55, or 5014-40-58). The virus stock was derived from NIH-3T3 mouse (Swiss) embryo cells infected with Moloney leukemia virus. It had been purified by double density gradient zonal centrifugation and contained 10^{11} to 10^{12} virus particles per milliliter. Protein content: 0.2–1.0 mg/mL for lot no. 719-24-9 and 810-71-8, 0.6 mg/mL for lot no. 5014-40-55 and 5014-40-58, and 1.7 mg/mL for lot no. 5004-26-56.

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The virus stocks were stored at −70 °C. Prior to use, 8% glycerol was added, and the virus samples were further kept at 4 °C during the course of the experiments.

Reference Compounds. Ethidium bromide (2,7-diamino-10-ethyl-9-phenylphenanthridium bromide) was obtained from Calbiochem (Los Angeles, Calif.), whereas tilorone dihydrochloride [2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one dihydrochloride] was supplied by Richardson-Merrell Inc. (Cincinnati, Ohio). Berenil (diminazene diaceturate) was a product of Hoechst A.G. (Frankfurt/Mainz, Federal Republic of Germany), whereas stilbamidine isethionate (4,4′-stilbenedicarboxamidine diisethionate) and pentamidine isethionate (4,4′-diamidino-α,ω-diphenoxypentane isethionate) were products of May and Baker Ltd (Dagenham, England).

Heat-Induced Formation of α,β-Unsaturated Nucleoside Dialdehydes and Their Activity with Adenosine Deaminase

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Application of heat to aqueous solutions of nucleoside dialdehydes (periodate-oxidized nucleosides) affords the corresponding α,β-unsaturated aldehydes. The reaction was first discovered during studies with adenosine deaminase and was initially investigated enzymatically until the nature of the chemical transformation was determined. A UV peak at 230–250 nm, characteristic of the α,β-unsaturated aldehyde group, was obtained by difference spectroscopy and affords a more practical means to study the reaction. Adenosine dialdehyde, obtained by periodate oxidation of adenosine, afforded the same product upon heating as obtained by periodate oxidation of 9-(5-deoxy-β-D-*erythro*-pent-4-enofuranosyl)adenine. Further proof of identity was obtained by reduction of these compounds with sodium borohydride and comparison of the dialcohols obtained to each other and to a known unsaturated dialcohol. Heating of nucleoside dialdehydes at any time is not recommended. The exact composition of nucleoside dialdehydes used in previous and current biological studies is open to question.

Nucleoside dialdehydes have potential use as chemotherapeutic agents for the treatment of neoplastic diseases.[2-5] They are obtained by periodate oxidation of nucleosides.[3,6,7] Especially important is inosine dialdehyde (1, Chart I), which has been tested clinically and for which the results of a phase I study have been published.[2] Although represented as structures like 1, it is generally recognized that nucleoside dialdehydes are really complex equilibrium mixtures of cyclic and acyclic hydrates and hemiacetals.[8-10] In fact, spectroscopic techniques do not even detect the aldehyde groups in solution,[9,11] a situation akin to that of reducing sugars, such as glucose, which are similarly defined as poly(hydroxyaldehydes).

While studying the deamination of adenosine dialdehyde (2) as catalyzed by adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) from calf intestinal mucosa, it was observed that application of a small amount of heat from a steam bath to aid in dissolution of the dialdehyde in phosphate buffer resulted in erratic kinetic results. Whereas 2 was a weak substrate for the enzyme, substrate activity was lowered appreciably or lost entirely upon heating. Moreover, 2, which was an extremely poor competitive inhibitor, became a rather strong inhibitor after heating. It appeared that a transition was taking place. This is a serious problem because the solubilities of some of the nucleoside dialdehydes are rather low and require heat to effect dissolution. Furthermore, preparative procedures for nucleoside dialdehydes often include extraction of the product from salts with hot alcohols and drying of the lyophilized powders in a drying pistol with heat.[9,11-13]

Table I. Comparison of the Inhibitor Constants of Solutions of Nucleoside Dialdehydes, Before and After Heating, with Adenosine Deaminase[a]

nucleoside dialdehyde	K_I, μM: before heating[b]	K_I, μM: after heating[c]
1		
2	520	5
3	310	8
4	28	30
5	780	800
6	46	30
7	430	24
8		69
9		38
10	150	20
11	370	21
12		
13	160	100

[a] 0.05 M sodium phosphate buffer (pH 7.0). [b] Most values are reported in ref 7 and 15. [c] Heated to 100 °C; see text for details.

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